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CD4$^+$ T-cell activation is differentially modulated by bacteria-primed dendritic cells, but is generally down-regulated by n-3 polyunsaturated fatty acids

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Summary

Appropriate activation of CD4$^+$ T cells is fundamental for efficient initiation and progression of acquired immune responses. Here, we showed that CD4$^+$ T-cell activation is dependent on changes in membrane n-3 polyunsaturated fatty acids (PUFAs) and is dynamically regulated by the type of signals provided by dendritic cells (DCs). Upon interaction with DCs primed by different concentrations and species of gut bacteria, CD4$^+$ T cells were activated according to the type of DC stimulus. The levels of CD80 were found to correlate to the levels of expression of CD28 and to the proliferation of CD4$^+$ T cells, while the presence of CD40 and CD86 on DCs inversely affected inducible costimulator (ICOS) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) levels in CD4$^+$ T cells. For all DC stimuli, cells high in n-3 PUFAs showed reduced ability to respond to CD28 stimulation, to proliferate, and to express ICOS and CTLA-4. Diminished T-cell receptor (TCR) and CD28 signalling was found to be responsible for n-3 PUFA effects. Thus, the dietary fatty acid composition influences the overall level of CD4$^+$ T-cell activation induced by DCs, while the priming effect of the DC stimuli modulates CD80, CD86 and CD40 levels, thereby affecting and shaping activation of acquired immunity by differential regulation of proliferation and costimulatory molecule expression in CD4$^+$ T cells.

Keywords: costimulation; dendritic cell; lipid; nutritional immunology; signalling; T cell

Introduction

CD4$^+$ T-cell activation occurs through the interaction of T cells with antigen-presenting cells (APCs) and is mediated by the antigen–receptor complex (TCRξ/CD3) and costimulatory molecules, especially the positive costimulators CD28 and inducible costimulator (ICOS), and the negative costimulator cytotoxic T-lymphocyte antigen-4 (CTLA-4; CD152).[1] Efficient T-cell activation depends not only on the type of APC stimulation, but also on the effectiveness in clustering of key elements in the immunological synapse, which is mandatory for efficient signal transduction through the plasma membrane. Accordingly, the composition of plasma membrane lipids plays an important modulating role in T-cell activation, as supported by recent reports on the differential influence of lipids on the capacity of the immune system to become activated.[2–6] A principal characteristic of long-chain n-3 polyunsaturated fatty acids (PUFAs) is the potential to down-regulate many aspects of immune responses, including lymphocyte proliferation, cytokine responses and antigen presentation on APCs.[7–12] One central element in studies revealing T-cell-suppressive effects of n-3 PUFAs seems to be engagement of the T-cell receptor (TCR) and/or CD28.[7,13,14] Thus, it appears that signalling cascades induced through the TCR and/or CD28 may be affected by the incorporation of n-3

Abbreviations: APC, antigen-presenting cell; CTLA-4, cytotoxic T-lymphocyte antigen-4; DC, dendritic cell; FA, fatty acid; FCS, fetal calf serum; ICOS, inducible costimulator; iDC, immature DC; IL, interleukin; LPS, lipopolysaccharide; MHC class II, major histocompatibility complex class II; MLN, mesenteric lymph node; MUFA, monounsaturated FA; PBS, phosphate-buffered saline; PE, phycoerythrin; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SPL, spleen; TCR, T-cell receptor; Th, T helper.

PUFAs into cellular lipids. Disruption of lipid raft integrity has been suggested as one underlying mechanism for n-3 PUFA-mediated T-cell inhibition, as a result of the displacement of signalling proteins [e.g. linker for activation of T cells (LAT)] from the raft regions of the membranes.[4,15,16] Although previous studies focusing on the effects of n-3 PUFAs on T cells have identified mechanisms by which the n-3 PUFAs may act at the cellular level, the correlations between APC signal strength and the presence of higher levels of dietary n-3 PUFAs, and hence the amounts of cellular n-3 PUFAs, in modulating $CD4^+$ T-cell responsiveness and expression of costimulatory molecules remain to be defined.

The type and level of costimulatory molecule expression on activated $CD4^+$ T cells are considered as key elements for the activation of adaptive immune responses. For B-cell activation, one important mechanism is engagement of B-cell-derived CD40 and CD40 ligand (CD40L) on activated $CD4^+$ T cells. Notably, the T-cell costimulatory molecule ICOS is recognized to play an essential role in CD40L regulation,[17] but our knowledge of the modulation of ICOS displayed on $CD4^+$ T cells is limited. ICOS is a member of the CD28 family,[18] but in contrast to CD28, ICOS is not constitutively expressed on naïve T cells and does not costimulate the production of interleukin (IL)-2. Moreover, ICOS differs from CD28 and CTLA-4 in that it binds to B7RP-1 [ICOS-ligand (ICOS-L), B7h,[19]] instead of CD80 (B7·1) and CD86 (B7·2). ICOS is implicated in some human autoimmune diseases, as increased ICOS expression is found on T cells from, for example, patients with rheumatoid arthritis[20] or systemic lupus erythematosus.[21] In mice, ICOS expression has been related to both T helper (Th)1-mediated and Th2-mediated diseases, with ICOS-deficient mice being resistant to the development of rheumatoid arthritis,[22] whereas blockade of ICOS in an allergic airway disease model leads to decreased Th2-mediated inflammation as well as to reduced serum immunoglobulin E (IgE) production.[23]

The T-cell costimulatory molecule, CTLA-4, plays a critical role in the down-regulation of T-cell activation. CTLA-4 ligation to B7 results in the inhibition of T-cell activation by blocking cytokine production and cell cycle progression.[24] In general, B7 family-related costimulatory molecules are considered to modify the TCR signal, either by enhancing (CD28/ICOS) or abrogating (CTLA-4) it.[25] Hence, because of the importance of these costimulatory molecules for T-cell fate as well as their dependency of proper raft formation, modulation of CD28, ICOS and CTLA-4 expression levels by cellular lipids might be a way to selectively regulate T-cell responses by the diet, thereby affecting adaptive immunity.

In this study, we examined the influence of an n-3 PUFA-rich diet on the ability of $CD4^+$ T cells to respond to an exogenous signal provided by dendritic cells (DCs) primed with different bacteria, corresponding to a variety of microbe-associated molecular patterns, and compared the effects with that obtained by TCR/CD28 cross-linking, thus assessing the direct effect of lipids on T cells when activated through the TCR and CD28. Our data provided evidence that $CD4^+$ T cells are distinctly regulated by bacteria-primed DCs, giving rise to variations in proliferation as well as to CD28, ICOS and CTLA-4 expression levels, but that dietary n-3 PUFA supplementation leads to generally reduced responsiveness of $CD4^+$ T cells as a result of diminished TCR/CD28 signalling in $CD4^+$ T cells.

Materials and methods

Mice

All mice were purchased from Taconic (Lille Skensved, Denmark) and were used at 8–12 weeks of age. At the onset of each experiment, BALB/c mice were transferred from a standard chow diet (Altromin 1324; Altromin, Lage, Germany) to one of two experimental diets composed of (wt%): 56% corn-starch (Bestfood Nordic, Skovlunde, Denmark), 20% casein (Miprodan milk proteins; Arla Foods, Viby, Denmark), 10% sucrose (Danisco Sugar, Copenhagen, Denmark), 5% salt mixture including trace elements (prepared in-house), 4% cellulose powder (MN 100; Frisenette, Ebeltoft, Denmark), 0·5% choline chloride (Merck, Darmstadt, Germany), 0·5% vitamin mixture (prepared in-house) and 4% fat, differing in the fatty acid (FA) compositions as described in Table 1. The oils were composed of melted coconut and safflower oil [saturated fatty acid (SFA) diet], and fish oil (Eskimo-3 Food; Cardinova AB, Uppsala, Sweden) and safflower oil (n-3 PUFA diet). Oils were purchased from a local distributor and stored in the dark at 4° until use. The diets contained sufficient essential PUFAs. The fish oil (Eskimo-3 Food; Cardinova AB) is stabilized with a mixture of natural antioxidants (Pufanox®; Cardinova AB) and contains 4·5 IU/g of vitamin E. Mice were given experimental diets for 2 weeks. Before each experiment, the diets were freshly prepared and then frozen. All animal studies were approved by The Danish Animal Experiments Inspectorate and were carried out according to the guidelines of 'The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purposes'.

Antibodies, cytokines and reagents

Purified anti-CD3 (NA/LE, clone 145-2C11) and purified anti-CD28 (NA/LE, clone 37·51) were purchased from BD Biosciences (Franklin Lakes, NJ). A PKH26 Red Fluorescent Cell Linker kit was obtained from Sigma-Aldrich (St Louis, MO) and used for PKH26 staining of $CD4^+$ T cells. The magnetic antibody cell sorting (MACS) CD4 isolation kit was from Miltenyi (Bergish Gladbach, Germany).

Table 1. Fatty acid composition of experimental diets (wt %)[1]

	n-3 PUFA	SFA
C6:0	ND	0·7 ± 0·0
C8:0	ND	6·8 ± 0·6
C10:0	ND	5·0 ± 0·3
C12:0	ND	34·3 ± 0·2
C14:0	5·9 ± 0·1	13·0 ± 0·2
C16:0	15·7 ± 0·1	8·9 ± 0·6
C16:1	6·3 ± 0·2	0·1 ± 0·0
C18:0	3·4 ± 0·2	3·5 ± 0·2
C18:1 (n-9)	10·3 ± 0·2	8·5 ± 0·3
C18:1 (n-7)	2·4 ± 0·1	0·2 ± 0·0
C18:2 (n-6)	19·4 ± 0·2	18·5 ± 1·1
C18:3 (n-3)	0·7 ± 0·0	ND
C20:1 (n-9)	0·9 ± 0·0	ND
C18:4 (n-3)	2·4 ± 0·1	ND
C20:4 (n-3)	0·6 ± 0·0	ND
C20:5 (n-3)	14·1 ± 0·9	ND
C21:5 (n-3)	1·1 ± 0·2	ND
C22:6 (n-3)	9·4 ± 0·7	ND
∑SFA	26·1 ± 0·9	72·3 ± 0·1
∑MUFA	25·1 ± 0·6	9·1 ± 0·9
∑n-6 PUFAs	19·6 ± 0·0	18·5 ± 1·1
∑n-3 PUFAs	29·2 ± 1·5	ND

[1]Only fatty acids (FAs) of > 0·5 wt% are listed. Data represent the mean ± standard deviation (SD) from three experiments.
MUFA, monounsaturated fatty acid; ND, not detectable; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Antibodies used for flow cytometry were: allophycocyanin-conjugated CD4 (clone RM4-5), phycoerythrin (PE)-conjugated CTLA-4 (clone UC10-4B9), PE-conjugated ICOS (clone 7E.17G9), PE-conjugated CD86 (clone GL1), allophycocyanin-conjugated CD11c (clone HL3) (all BD Biosciences, San Diego, CA), PE-conjugated CD28 (clone 37·51, eBioscience, San Diego, CA), PE-conjugated major histocompatibility complex class II (MHC class II) (clone NIMR-4), PE-conjugated CD40 (clone 1C10) and PE-conjugated CD80 (clone 1G10) (all Southern Biotech, Birmingham, AL). Murine immunoassay kits [interferon-γ (IFN-γ), IL-10 and IL-5] were all obtained from R&D Systems (Minneapolis, MN).

Bacterial strains

The bacterial strains were all gut flora-derived and selected according to specific immunoregulatory properties on murine DCs from a larger screening study of Gram-positive strains performed in our group (Zeuthen *et al.*, manuscript in preparation). The bacteria were grown, harvested and killed by exposure to ultraviolet (UV) light, as described in Zeuthen *et al.*[26] The dry weight of bacteria was determined by freeze-drying aliquots and correcting for phosphate-buffered saline (PBS) content. Bacteria were stored at −80°.

Generation and culture of DCs

DCs were generated from bone marrow cells derived from C57BL/6 in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) (15 ng/ml), as previously described.[27] On day 8, cells (approximately 1×10^7 cells/petri dish) were stimulated for 18 hr with 0·1 or 100 μg/ml of UV-killed gut flora-derived bacteria or lipopolysaccharide (LPS) (1 μg/ml final concentration of *Escherichia coli* O26:B6; Sigma-Aldrich), without the addition of fresh GM-CSF to culture plates. DCs cultured with culture medium alone were termed immature DCs (iDCs). The purity of DCs was > 90%, as determined by CD11c staining followed by flow cytometry.

$CD4^+$ T-cell activation

$CD4^+$ T cells were positively selected from spleen (SPL) and mesenteric lymph nodes (MLNs) using anti-CD4 microbeads (MACS; Miltenyi), according to the manufacturer's instructions, and were more than 85% (SPL) and 98% (MLN) pure, as assessed by flow cytometry. For PKH26 labelling, $CD4^+$ SPL T cells were diluted to 1×10^7 cells/ml in 2 μM PKH/diluent C (Sigma-Aldrich), incubated first for 3 min, then for 1 min with fetal calf serum (FCS) (1 : 1, v/v) and then extensively washed in culture medium containing 10% FCS. For proliferation assays using stimulation with anti-CD3/CD28, $CD4^+$ SPL T cells were seeded at 2×10^5 cells per well into round-bottomed 96-well plates (Nunc, Roskilde, Denmark) containing plate-bound anti-CD3 (2 μg/ml), with or without anti-CD28 (5 μg/ml) in complete medium in which 2% heat-inactivated autologous, diet-dependent serum replaced FCS. Before each experiment, a pool of autologous serum from each dietary group was obtained by heart puncture during anaesthesia; the serum was collected into sterile, non-heparized tubes, centrifuged at 3000 ***g*** for 10 min and heat inactivated at 56° for 30 min. After incubation for 72 hr, each culture was pulsed with [^{3}H]thymidine (0·25 μCi; Amersham, Bucks, UK) for 18 hr to assess proliferative activity by liquid scintillation counting (Tri-Carb®; Packard, Meriden, CT). The change in counts per minute (Δc.p.m.) was calculated by subtracting the average of triplicate cultures of stimulated cells from that of control cells. To measure total cell divisions, PKH-labelled $CD4^+$ SPL T cells were cultured as described above and, after 4 days of incubation, the cells were washed once, resuspended in PBS/1% azide and analysed using flow cytometry.

For DC-induced T-cell proliferation, graded numbers of bacteria-treated DCs or iDCs were cultured with allogenic PKH-labelled $CD4^+$ SPL T cells (10^5 cells per well in 96-well round-bottom plates, corresponding to DC : T-cell ratios of 1:10, 1:20 and 1:40) for 5 days in

complete medium in which 1% autologous, diet-dependent murine serum replaced FCS. The total number of $CD4^+$ T-cell divisions was recorded using flow cytometry.

Flow cytometry

For surface staining of $CD4^+$ T cells and DCs, cells were incubated with antibody to FcR (24G2) and stained with the appropriate antibodies or isotype controls in PBS containing 0·15% (v/v) sodium azide and 1% FCS. For intracellular staining of CTLA-4, $CD4^+$ T cells were fixed in PBS containing 4% methanol-free formaldehyde, washed in PBS containing 0·1% saponin and 0·5% bovine serum albumin (BSA) (both from Sigma), blocked with anti-FcR and incubated with appropriate antibodies or isotype control in PBS containing 0·1% saponin and 0·5% BSA. Cells were analysed on a FACSarray flow cytometer (BD Biosciences). Data analyses were performed using FCS EXPRESS (Version 3; De Novo Software, ON, Canada).

Cytokine determinations

Supernatants collected after 48 hr of anti-CD3/CD28-induced activation of SPL or MLN $CD4^+$ T cells were assayed for IFN-γ, IL-10 and IL-5 by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions.

Fatty acid analysis

Lipids were extracted from cells as previously described.[28] Phospholipids (PL) were isolated from the cell extract, using preparative thin-layer chromatography (TLC) with a solvent system consisting of heptane : 2-propanol : acetic acid (95:5:1, v/v/v). Lipid spots were visualized by spraying with 2′,7′-dichlorfluorescein (1 g in 500 ml of EtOH), scraped from the plates and FA methyl esters were prepared essentially as described previously.[28] The FA methyl esters were separated on a 50 m SP-2380 column (Sigma-Aldrich, Brøndby, Denmark) in a Hewlett Packard 6890 gas-chromatograph (GC), in split mode using helium as the carrier gas. The GC settings were as follows: injector temperature, 250°; split-ratio, 20:1; carrier-flow, 1·2 ml/min; detector temperature, 270°; air flow in detector, 400 ml/min; hydrogen flow, 30 ml/min. The temperature started at 50° and was increased to 140° at a rate of 30°/min; this temperature was maintained for 2 min, and hereafter the temperature was increased to 220° at a rate of 2°/min and the oven was maintained at 220° for 5 min before the temperature was increased to 250° at a rate of 10°/min. The final temperature was maintained for 17 min. FA methyl esters was identified using authentic standards. Calibration was performed using a quantitative standard (Nu-Check Prep Inc., Elysian, MN).

Statistical analysis

Data were tested for statistical significance using an unpaired *t*-test or a two-way analysis of variance (ANOVA), as described in the figure legends. Bivariate correlations were performed with Spearman's correlation analysis. All data were analysed using GRAPHPAD PRISM software (version 4·03; GraphPad Software, La Jolla, CA). A *P*-value of $\leq 0{\cdot}05$ was considered significant.

Results

Changes in n-3 PUFA content affect CD3/CD28-induced $CD4^+$ T-cell activation

To examine the specific effect of changing the dietary amount of n-3 PUFAs on the cellular FA compositions in $CD4^+$ T cells, we fed two different diets (Table 1) *ad libitum* to BALB/c mice for 14 days. The phospholipid FA composition of SPL $CD4^+$ T cells revealed that the dietary n-3 PUFAs was incorporated into cell membranes at the expense of n-6 PUFAs, with similar amounts of total cellular PUFAs being present in the two dietary groups, leading to threefold differences in the n-6 : n-3 PUFA ratios between the two groups (Table 2). Conversely, the differences between SFA and monounsaturated fatty acid (MUFA) contents in the two diets were not reflected in the phospholipid FA composition of the $CD4^+$ T cells from the two groups.

The effect of the n-3 PUFA diet versus the SFA diet on $CD4^+$ T-cell activation was initially examined by cross-linking with anti-CD3 and anti-CD28 (Fig. 1). The proliferative capacity of $CD4^+$ T cells was significantly lower in cells with a high n-3 PUFA content, compared withcells low in n-3 PUFAs (Fig. 1a). Tracking the total number of cell divisions over 4 days confirmed that cells with a high n-3 PUFA content had markedly decreased numbers of cell divisions compared to cells with a low n-3 PUFA content (Fig. 1b, $P < 0{\cdot}0001$). Moreover, incorporation of n-3 PUFAs into cell membranes was found to significantly reduce the production of IFN-γ and IL-10, whereas the production of IL-5 was unaffected (Fig. 1c).

n-3 PUFAs diminish signalling through TCR and CD28 in activated $CD4^+$ T cells

As the effects of n-3 PUFAs on $CD4^+$ T cells are particularly clearly exposed upon TCR and CD28 activation, as shown in Fig. 1, we examined whether the expression and/or function of costimulatory molecules, especially CD28, on $CD4^+$ T cells is directly influenced by n-3 PUFAs.

In accordance with earlier findings,[14] surface expression of CD28 on resting SPL $CD4^+$ T cells was found not to be affected by changes in the amounts of cellular n-3 PUFAs (Fig. 2a). The same was observed for resting $CD4^+$ T

Table 2. Fatty acid composition (in mol %) of $CD4^+$ T cells from mice fed the indicated diets[1]

	n-3 PUFA	SFA
C14:0	0·6 ± 0·1	1·1 ± 0·4
C16:0	29·4 ± 3·3	29·1 ± 3·7
C16:1 (n-7)	1·4 ± 0·4	1·3 ± 0·3
C18:0	19·2 ± 1·8	18·2 ± 1·9
C18:1 (n-9)	7·0 ± 0·2	7·5 ± 0·4
C18:1 (n-7)	3·0 ± 0·6	3·7 ± 0·5
C18:2 (n-6)	9·6 ± 0·5	7·1 ± 1·9
C20:2 (n-6)	0·8 ± 0·3	1·0 ± 0·2
C20:3 (n-6)	1·3 ± 0·3	1·1 ± 0·1
C20:4 (n-6)	12·9 ± 2·7	20·4 ± 3·3[2]
C20:5 (n-3)	1·8 ± 0·9	ND
C22:4 (n-6)	0·6 ± 0·2	2·6 ± 0·4
C22:5 (n-6)	0·2 ± 0·0	0·8 ± 0·2
C22:5 (n-3)	3·8 ± 0·5	1·2 ± 0·6
C22:6 (n-3)	8·0 ± 0·9	4·3 ± 1·5
∑SFA	49·3 ± 3·6	48·4 ± 4·0
∑MUFA	11·8 ± 0·5	12·7 ± 0·9
∑n-6 PUFAs	25·4 ± 3·1	33·0 ± 3·1[2]
∑n-3 PUFAs	13·5 ± 1·3	5·5 ± 1·0[2]
n-6/n-3 PUFAs	1·9 ± 0·2	6·0 ± 0·5

[1]$CD4^+$ T cells were isolated from spleens upon 14 days feeding with the diets. Data are fatty acid (FA) compositions in mole percentages of major fatty acids (> 0·5 mol%) from the phospholipid fraction, and represent the mean ± standard deviation (SD) from three experiments each performed with a pool of cells from seven mice.

[2]$P < 0·001$ by two-way analysis of variance (ANOVA) and Bonferroni's *post hoc* test.

MUFA, monounsaturated fatty acid; ND, not detectable; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

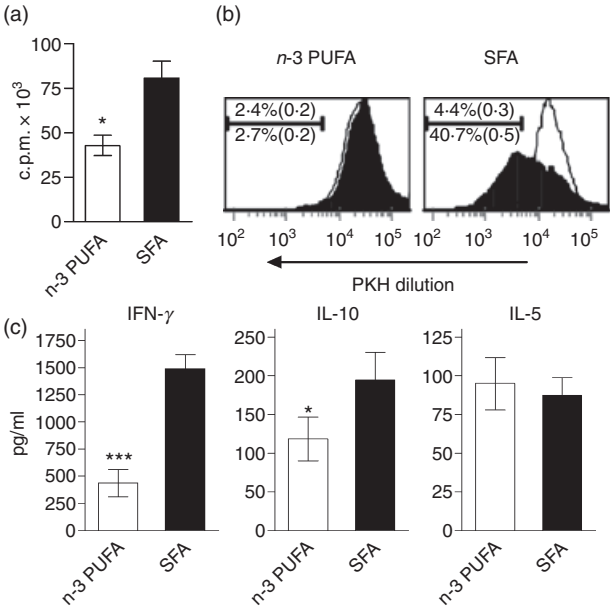

Figure 1. n-3 Polyunsaturated fatty acid (PUFA) incorporation into cell membranes inhibits CD3/CD28-induced $CD4^+$ T-cell activation. (a) Proliferation of $CD4^+$ spleen (SPL) T cells isolated from BALB/c mice fed diets containing high levels of saturated fatty acids (SFAs) or n-3 PUFAs for 14 days. $CD4^+$ T cells (2×10^5 cells) were cultured in 96-well round-bottom plates in the presence of plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (5 μg/ml) for 4 days. During the last 18 hr of incubation, [^{3}H]thymidine was added. Data represent means ± standard deviation (SD) from four experiments, each using $CD4^+$ T cells pooled from seven mice. (b) PKH-labelled $CD4^+$ T cells from mice with the two dietary treatments were cultured for 4 days, as described above. Proliferation of $CD4^+$ T cells was measured using flow cytometry to assess the PKH dilution in dividing cells. Percentages of dividing cells are given below (anti-CD3/anti-CD28-stimulated, black) or above (unstimulated, black line), respectively. The SDs from four experiments are given in parentheses. Histograms are one representative result of four experiments. (c) Cytokine production in supernatants [interferon-γ (IFN-γ), interleukin (IL)-10 and IL-5] from $CD4^+$ T cells activated as described in panel a and measured using enzyme-linked immunosorbent assay (ELISA). Data are means ± SD ($n = 4$). $^{***}P < 0·001$, $^{*}P < 0·05$, analysed using the unpaired *t*-test.

cells from MLNs (Fig 2a). To clarify whether the lower activation ability of $CD4^+$ T cells with higher n-3 PUFA content was caused by reduced signalling via the TCR alone, or if signalling via both the TCR and CD28 was implicated, we added either anti-CD3 alone or anti-CD3 and anti-CD28 together. The effects on cellular proliferation (Fig. 2b) and IFN-γ production from SPL $CD4^+$ T cells (Fig. 2c), showed that higher cellular n-3 PUFA contents reduced signalling through the TCR and completely abolished any additive effect via CD28. Cells high in n-3 PUFAs were also found to have a lower expression level of the costimulator ICOS upon anti-TCR and anti-CD28 activation (Fig. 2d), indicating that co-upregulation of ICOS as a result of potent TCR and CD28 activation is, to some extent, reduced in n-3 PUFA-rich $CD4^+$ T cells.

Proliferation is dynamically regulated by the FA content and DC signal strength

To further address the effect of n-3 PUFAs on $CD4^+$ T-cell activation at the level of TCR and costimulatory molecule regulation, we co-cultured $CD4^+$ T cells, from the two dietary groups, with allogenic DCs. The DCs were primed with different concentrations and genera of gut bacteria in order to study the effect of the strength of the DC signal on the $CD4^+$ T-cell activation capacity. Gut bacteria were selected because they expose different microbe-associated molecular structures and have diverse priming effects on DCs. Two Gram-positive species of bacteria [*Lactobacillus acidophilus* X37 (*L. acidophilus* X37) and *Bifidobacterium longum* Q46 (*B. longum* Q46)] and one Gram-negative gut commensal [*Escherichia coli* Nissle 1917 (*E. coli* Nissle)] were used in the study. DCs matured by LPS were included in the study for comparison. Upon stimulation with bacteria or with LPS, the surface expression of MHC class II, CD80, CD86

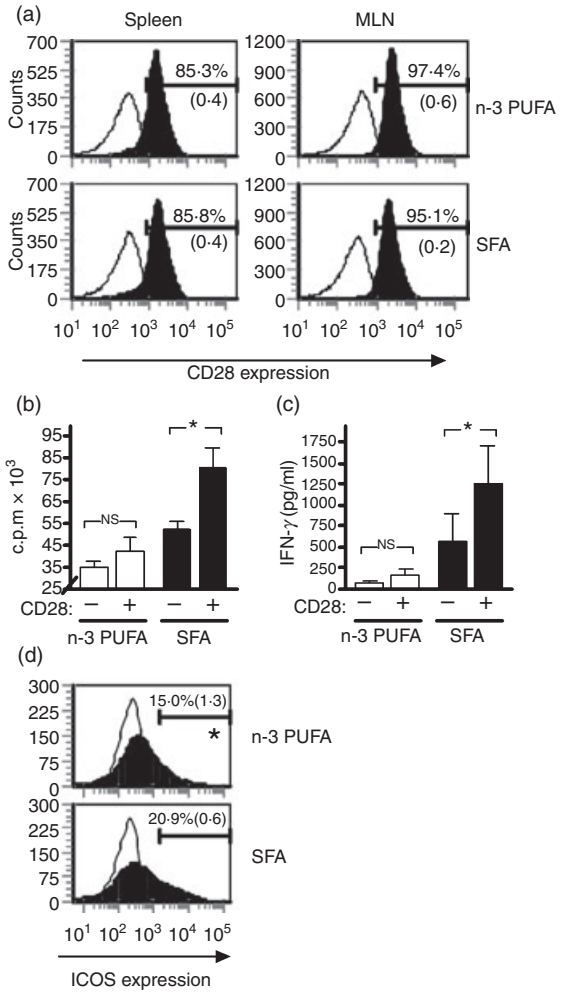

Figure 2. Incorporation of n-3 polyunsaturated fatty acids (PUFAs) in $CD4^+$ T cells reduces T-cell receptor (TCR)/CD28-induced signalling without affecting CD28 surface expression in resting cells. (a) $CD4^+$ T cells isolated from spleen (SPL) and mesenteric lymph nodes (MLNs) from BALB/c mice fed two different diets [n-3 PUFA or saturated fatty acids (SFAs)] for 14 days were surface stained with phycoerythrin (PE) anti-CD28 monoclonal antibody (mAb) (filled histograms), without previous activation, and analysed by flow cytometry. The mean percentage of positively stained cells are indicated above the marker. Histograms are representative of one of three experiments, whereas means and standard deviations (SDs) (given in parentheses below the marker) are based on three experiments. The empty histograms represent isotype-stained cells. Median fluorescence intensity (MFI) values for the groups are as follows: SPL, n-3 PUFA: 2091 ± 523, SFA: 2041 ± 150 (NS); MLN, n-3 PUFA: 2407 ± 163, SFA: 1942 ± 251 (NS). (b) Proliferation of SPL $CD4^+$ T cells after 4 days of culture with plate-bound anti-CD3 (2 μg/ml), with or without the addition of anti-CD28 (5 μg/ml), assessed as described in the legend to Fig. 1a. Data are means ± SD ($n = 4$). c.p.m., counts per minute. (c) Interferon-γ (IFN-γ) production measured using enzyme-linked immunosorbent assay (ELISA) in supernatants from SPL $CD4^+$ T cells cultured for 2 days as described for panel b. Data represent means ± SD ($n = 4$). (d) Inducible costimulator (ICOS) expression in day 2 SPL $CD4^+$ T cells activated with anti-CD3 and anti-CD28 (filled histograms); empty histograms correspond to the isotype control. The histograms representative of one of three experiments. The mean percentages of positively stained cells are indicated above the marker, and the SD is shown in parentheses ($n = 3$). The MFI for ICOS, n-3 PUFA: 2307 ± 145, and for SFA: 2941 ± 64 (*). *$P < 0{\cdot}05$ by unpaired t test, NS, non-significant.

and CD40 on DCs increased when exposed to a high concentration (100 μg/ml) of bacteria and 1 μg/ml of LPS, compared with exposure to a lower concentration (0·1 μg/ml) of bacteria, (Fig. 3). For *B. longum* Q46 and *L. acidophilus* X37 at 0·1 μg/ml, the expression of CD80, CD86 and CD40 was similar to that of DCs cultured with medium alone (iDC), in that low expression of all surface markers was observed (Fig. 3b). With regard to MHC class II expression, the two concentrations of *E. coli* Nissle induced a level of MHC class II similar to that induced by LPS and 100 μg/ml of *L. acidophilus* X37, whereas the two concentrations of *B. longum* Q46 and the low concentration of *L. acidophilus* X37 induced lower levels of MHC class II.

For all surface markers measured, *E. coli* Nissle and LPS were found to be the most potent stimulators. At the high concentration, *L. acidophilus* X37 was, however, more potent in up-regulating MHC class II and CD40 than *E. coli* Nissle (and LPS), the latter being more potent in up-regulating the B7 molecules. A higher expression level of the costimulatory molecules on DCs is assumed to directly reflect the strength of the signal provided to $CD4^+$ T cells.

The proliferation-inducing capacity of the differentially matured DCs on $CD4^+$ T cells was examined by culturing the $CD4^+$ T cells with increasing numbers of DCs. Notably, the number of cell divisions in DC-induced $CD4^+$ T cells was found to be highly dependent on the FA composition of the $CD4^+$ T-cell membrane ($P < 0{\cdot}0001$ between diets; two-way ANOVA comparing curve slopes from the two diet treatments with the DC treatments, Fig. 4a) and less dependent on the DC maturational level ($P = 0{\cdot}055$). Incorporation of n-3 PUFAs into the $CD4^+$ T-cell membrane caused a significant reduction in cell proliferation, with the highest proliferation of T cells being obtained with DCs treated with 100 μg/ml of *E. coli* Nissle. Notably, the proliferation induced in n-3 PUFA-rich cells with *E. coli* Nissle-primed DCs resembled that induced by iDCs in the SFA group (as illustrated by the slope values, Fig. 4a), clearly illustrating the reduced, but not abrogated, proliferative capacity of n-3 PUFA-rich cells. In the presence of a higher n-6 : n-3 PUFA ratio in the cell membrane, the greatest proliferation of $CD4^+$ T cells was induced by DCs treated with both concentrations of *E. coli*, as well as by the highest concentration of both lactobacilli strains and LPS. Correlations of the proliferation data (Fig. 4a) with the expression level of the DC surface markers (Fig. 3) revealed that high expression levels of CD80 and CD86 on DCs affect the cell division rate

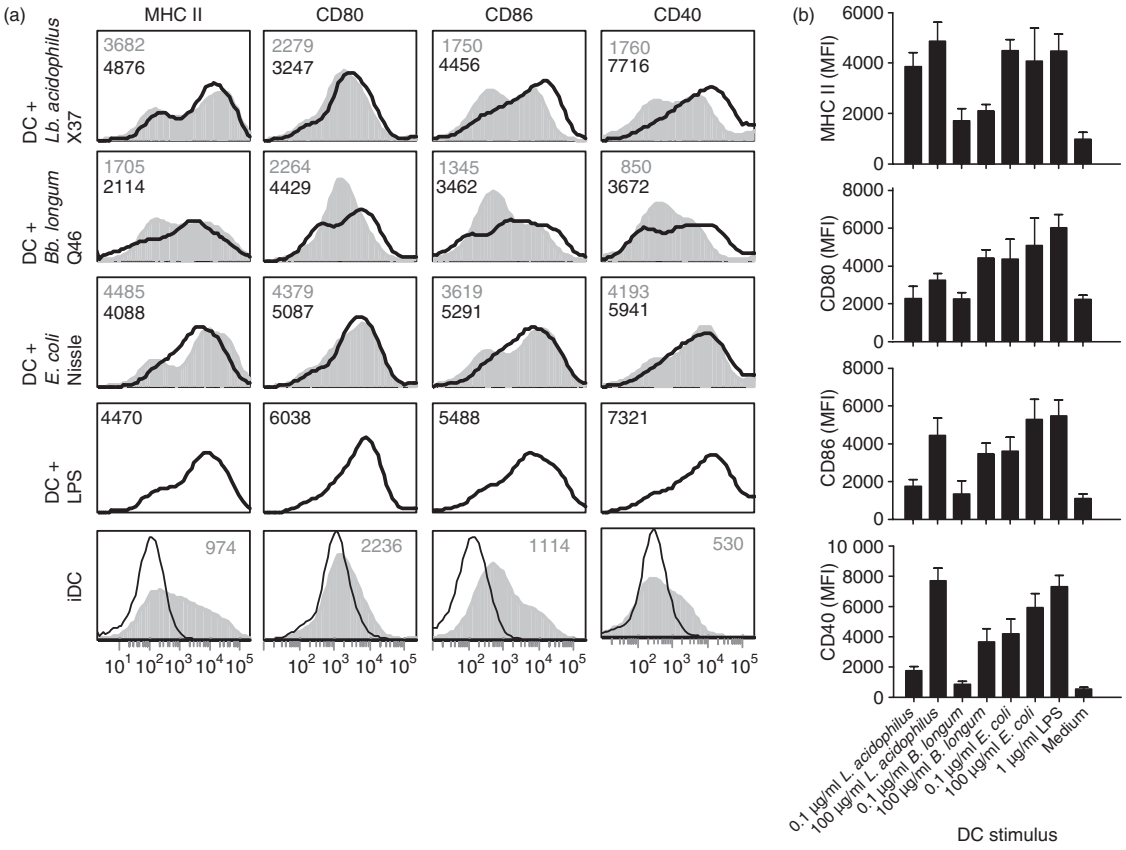

Figure 3. The expression level of major histocompatibility complex (MHC) class II and the costimulatory molecules CD80, CD86 and CD40 depends on the concentration and the genera of gut-derived bacteria. On day 8, immature dendritic cells (iDCs) (1×10^7 cells) were cultured with two concentrations (0·1 or 100 μg/ml) of either Gram-positive bacteria (*Lactobacillus acidophilus* X37 or *Bifidobacterium longum* Q46) or a Gram-negative bacterium (*Escherichia coli* Nissle) for 18 hr. Lipopolysaccharide (LPS) from *E. coli* O26:B6 was added at 1 μg/ml, or DCs were cultured with medium alone (iDCs). The surface phenotype of day 9 DCs was defined by flow cytometry after staining cells with phycoerythrin (PE)–MHC class II, PE–CD80, PE–CD86 or PE–CD40 monoclonal antibodies (mAbs), and gating on 20 000 viable DCs. (a) Histograms of surface expression levels of MHC class II, CD80, CD86 and CD40. The grey-shaded histogram corresponds to 0·1 μg/ml of bacteria, and empty histograms (black line) correspond to 100 μg/ml of bacteria. The isotype-stained cells are shown as thin histograms on the iDC plots. Numbers represent mean geometrical median fluorescence intensities (MFIs) of three experiments (grey numbers refer to grey histograms and black numbers to solid lines). Histograms correspond to the values obtained from one of three representative experiments. (b) Bar diagrams representing the variation in the data shown in panel a. Data are mean geometrical MFIs of three experiments + standard deviation (SD). The data on levels of surface marker expression on DCs are used to correlate to effects on $CD4^+$ T cells in terms of proliferation and costimulatory molecule expression and therefore no statistical analyses were performed on the DC marker expression display.

in $CD4^+$ T cells (correlation analysis not shown), while the DC expression levels of CD40 and MHC class II were found to be of minor importance with respect to proliferation-inducing capacity. In line with this, the iDCs induced the lowest proliferation in the $CD4^+$ T cells (from both dietary groups); a proliferation which resembled that induced by DCs stimulated with 0·1 μg/ml of the two lactobacilli strains.

Based on our observation of CD28 signalling to reduce the proliferation of n-3 PUFA-rich $CD4^+$ T cells upon anti-TCR and anti-CD28 cross-linking (Fig. 2) and the influence of the expression level of the CD28-ligands CD80 and CD86 on T cell division rate, we tested the effect of CD28 expression levels on $CD4^+$ T cells upon interaction with the differentially matured DCs. Notably, the percentage of CD28-positive cells was lower when $CD4^+$ T cells were co-cultured with a potent DC priming and proliferation-inducing stimulus (i.e. *E. coli* Nissle at 100 μg/ml) (Fig. 4b), presumably because of activation-induced internalization of CD28 upon activation through CD80/CD86, as previously reported.[29] Importantly, a lower membrane n-6 : n-3 PUFA ratio gave rise to higher CD28 expression in DC-activated SPL-derived $CD4^+$ T cells (Fig. 4b), indicating that n-3 PUFA-rich cells are slower to respond to CD80/CD86 signalling than cells with a threefold higher membrane n-6 : n-3 PUFA ratio.

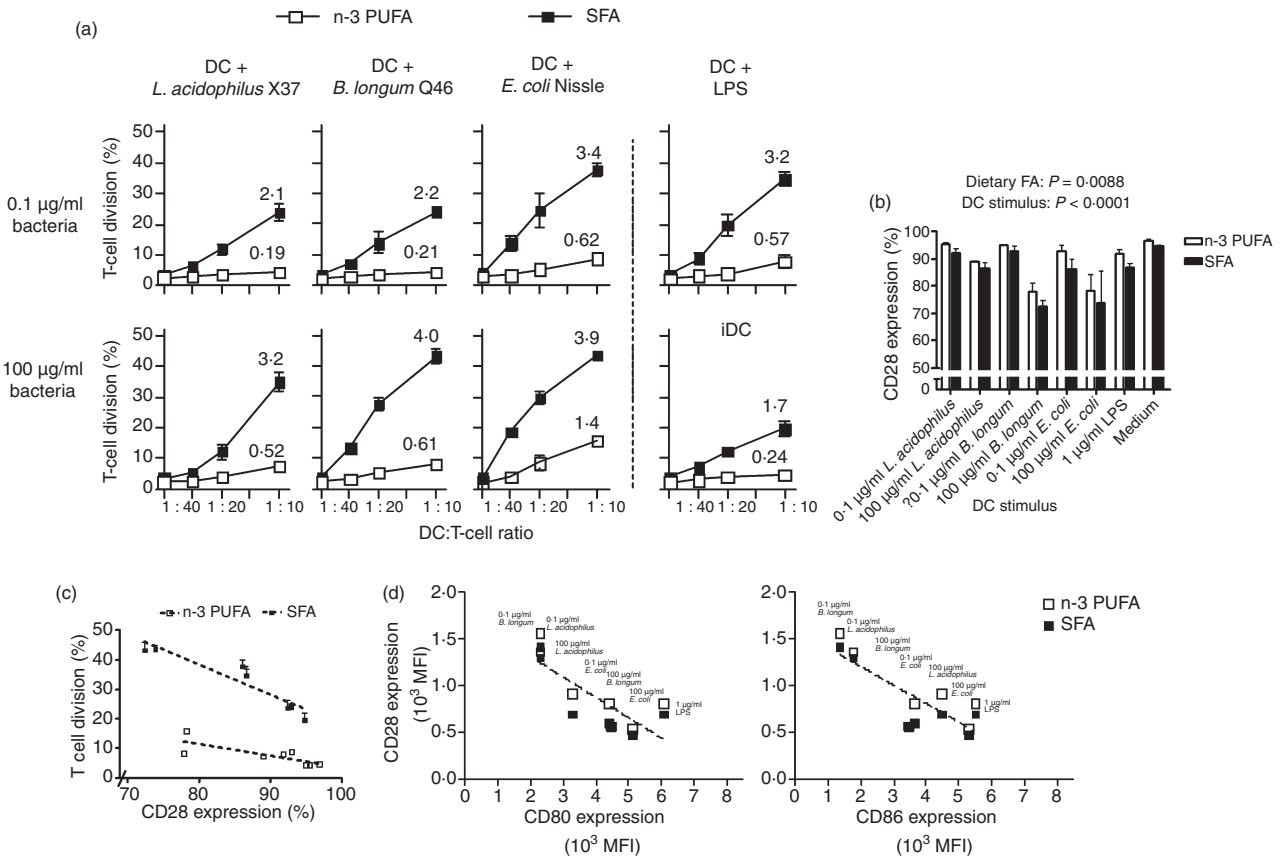

Figure 4. Proliferation of $CD4^+$ T cells depends on the dendritic cell (DC) signal strength and the T-cell responsiveness towards CD28 signalling. (a) Spleen (SPL) $CD4^+$ T cells were isolated from BALB/c mice fed either the n-3 polyunsaturated fatty acid (PUFA) or the saturated fatty acid (SFA) diet for 14 days, labelled with PKH and cultured at 10^5 cells per well with grading doses of bacteria-stimulated DCs, stimulated as described in Fig. 3. After incubation for 5 days, flow cytometry was performed to examine the dilution of PKH in order to assess $CD4^+$ T-cell divisions. The percentages of T-cell divisions were determined as described in Fig. 1b. Each data point represents the mean ± standard deviation (SD). The data represent three experiments. The number given above each proliferation curve indicates the slope of the curve ($\times 10^{-3}$), as analysed by linear regression analysis. (b) SPL $CD4^+$ T cells were co-cultured with *in vitro*-stimulated DC (at a ratio of 10:1, cultured as described in Fig. 3) for 5 days, and then analysed for CD28 expression by flow cytometry, gated on viable $CD4^+$ T cells. The data are mean + SD from two experiments. (c) Inverse linear correlation between the percentage of CD28-positive $CD4^+$ T cells (from Fig. 4b) and the cell-division ability for cells cultured at the DC : T-cell ratio of 1:10 (Fig. 4a). Linear regression lines are shown for both dietary groups: R^2 (n-3 PUFA), 0·57; and R^2 (SFA), 0·78. (d) Inverse linear correlations between CD28 expression on $CD4^+$ T cells [expressed as median fluorescence intensity (MFI)] and DC expression of CD80 and CD86 (from Fig. 3). Correlation coefficients, CD80: n-3 PUFA: $r = -0{\cdot}89$, $P = 0{\cdot}012$, SFA: $r = -0{\cdot}64$, $P = 0{\cdot}14$. CD86: n-3 PUFA: $r = -0{\cdot}68$, $P = 0{\cdot}11$, SFA: $r = -0{\cdot}50$, $P = 0{\cdot}27$. (a,b) Differences in proliferation or percentage of CD28 expression between the two dietary groups and the different DC stimuli were tested by two-way analysis of variance (ANOVA) and Bonferroni's *post hoc* test. *P*-values for the effects of diets and DC stimuli on proliferation and CD28-positive cells are given in the text (a) or in the figure (b). No interaction between the diets and the DC stimuli was observed.

Data for $CD4^+$ T cells from MLNs resembled those from SPL (data not shown). In line with this, an inverse correlation was found between the percentages of CD28-positive cells and the cell division for each DC treatment and diet group (Fig. 4c), indicating again that lack of CD28 responsiveness might explain the lower proliferation ability of n-3 PUFA-rich $CD4^+$ T cells. To test if the differences in CD28 expression on $CD4^+$ T cells after co-culture with differentially primed DC were caused by changes in B7 expression on DCs, we performed correlation analyses between CD28 expression levels versus CD80 and CD86 expression levels on DCs (Fig. 4d). No significant correlation was found for CD28 versus CD86, whereas if adjusted for diet treatment, a significant correlation was found for CD28 versus CD80 ($P = 0{\cdot}048$), but for the n-3 PUFA group only, if not adjusted. To study the effect of enhancing the dietary intake of n-6 PUFAs, we included a third diet group in one of the experiments,

in which the dietary content of n-6 PUFAs was increased to 40 wt%, while keeping the dietary n-3 PUFA content at 2 wt% (and the SFA content similar to that of the n-3 PUFA group). However, no further effects in regard to the cellular FA composition in $CD4^+$ T cells was observed, and the cell proliferation was similar to that of the SFA group (data not shown).

CTLA-4 and ICOS levels depend on n-3 PUFA content and DC signal strength

To evaluate the significance of the signal transmission capability in $CD4^+$ T cells on costimulatory molecule expression, induced through DCs, in the $CD4^+$ T cells harbouring different n-3 PUFA levels, we measured the expression of CTLA-4 and ICOS upon activation with the differentially primed allogenic DCs.

The percentage of ICOS-positive cells was shown to depend on both the DC signal and the cellular PUFA ratio, with the lowest percentages observed in cells with the highest n-3 PUFA content (Fig. 5a). Interestingly, we found an inverse correlation between the expression level of CD40 on DCs and the percentages of ICOS-positive SPL and MLN $CD4^+$ T cells (Fig. 5b, data not shown for MLN), thus indicating an indirect link between CD40L and ICOS expression on $CD4^+$ T cells.

With respect to CTLA-4, we measured the contents of cell-surface protein and intracellular protein simultaneously, in order to account for the total pool of CTLA-4, as CTLA-4 molecules are recognized to be

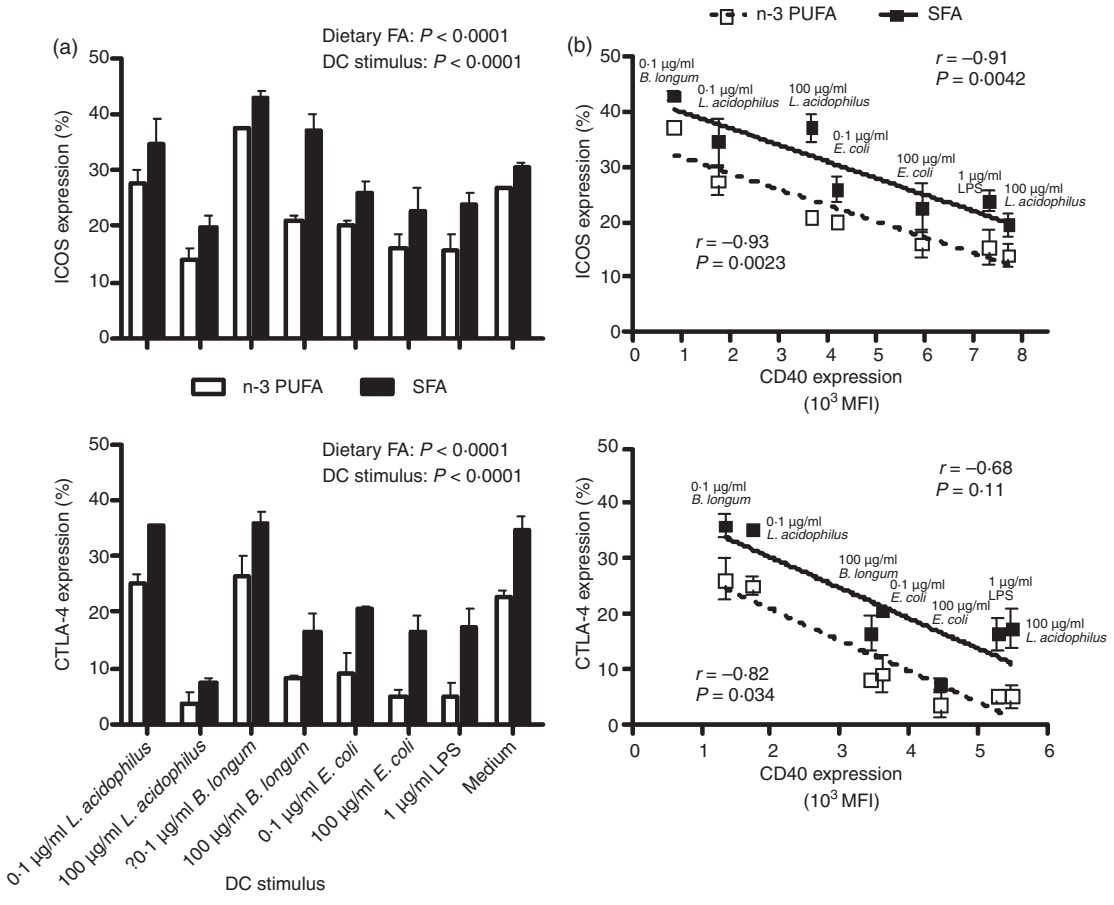

Figure 5. The expression level of the T-cell costimulatory molecules inducible costimulator (ICOS) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) is dependent on the CD40 level on dendritic cells (DCs), and is reduced in n-3 polyunsaturated fatty acid (PUFA)-rich $CD4^+$ T cells. Spleen (SPL) $CD4^+$ T cells, derived from mice fed diets high in n-3 PUFAs or saturated fatty acids (SFAs), were co-cultured with *in vitro*-stimulated DCs (at a ratio of 10:1, DCs were cultured as described in Fig. 3) for 5 days, stained with fluorescein-conjugated antibodies and analysed by flow cytometry with gating of viable $CD4^+$ T cells. (a) The percentage of ICOS- and CTLA-4-positive cells. Data are mean + standard deviation (SD) from two experiments. Differences in expression of T-cell surface markers between the two dietary groups and the different DC stimuli were tested by two-way analysis of variance (ANOVA) and Bonferroni's *post hoc* test. *P*-values are given in the boxes. No interaction between the diets and the DC stimuli was observed. (b) Linear regression analysis between CD40 or CD86 expression on DCs (Fig. 3) and mean percentage of ICOS-positive or CTLA-4-positive SPL $CD4^+$ T cells (Fig. 5a). Difference between dietary treatments, ICOS versus CD40: $P < 0{\cdot}0001$, CTLA-4 versus CD86: $P = 0{\cdot}0002$ (from linear regression analysis). Spearman's correlation coefficients are given below (n-3 PUFA) or above (SFA) the regression lines.

rapidly endocytosed from the surface.[30] The percentage of CTLA-4-positive cells was observed to follow the same tendencies as seen for ICOS, with lower percentages of positive SPL and MLN $CD4^+$ T cells in the n-3 PUFA group and upon stimulation with the most mature DCs (Fig. 5a). CTLA-4 expression on SPL and MLN $CD4^+$ T cells was found to correlate with the level of CD86 expression on DCs (Fig. 5b), as well as with CD40 expression (data not shown, n-3 PUFA, $r = -0{\cdot}93$, $P = 0{\cdot}0067$, SFA, $r = -0{\cdot}82$, $P = 0{\cdot}034$).

Collectively, a lower TCR/CD28 responsiveness in cells high in n-3 PUFAs was directly reflected by a lower percentage of ICOS-positive and CTLA-4-positive SPL and MLN $CD4^+$ T cells, while the DC signal strength, depending on the genera and concentration of bacteria, distinctly regulated the expression level of CD28, CTLA-4 and ICOS on $CD4^+$ T cells.

Discussion

$CD4^+$ T cells are central for activation of the acquired immune system, and the capacity of $CD4^+$ T cells to respond to signals provided by APCs is critical for the resulting immunity. Here, we examined the influence of the dietary and cellular lipid profile and the DC signal strength on the ability of $CD4^+$ T cells to express the co-stimulatory molecules CD28, ICOS and CTLA-4 and to proliferate after interaction with differentially primed DCs. Such information will provide us with basic knowledge into the regulatory effects of dietary lipid composition on $CD4^+$ T-cell responses.

Our use of differentially matured DCs permitted distinction between the influence of the DC signal strength and the cellular lipid profile for $CD4^+$ T-cell activation, a dynamic inter-relationship that has not yet been described. We found that the capacity of $CD4^+$ T cells to respond to an exogenous signal provided by DCs was greatly affected by the potency of the DC stimuli (particularly exemplified by differences in the expression of CD80, CD86 and CD40), resulting in diverse regulation of $CD4^+$ T-cell proliferation and expression of all costimulatory molecules investigated (CD28, CTLA-4 and ICOS). Importantly, for all DC stimuli, we found that the cellular PUFA ratio affected the activation ability of $CD4^+$ T cells, with a cellular n-6 : n-3 PUFA ratio of 2 requiring a larger threshold in order to become activated compared to cells with an n-6 : n-3 PUFA ratio of 6. The cellular n-6 : n-3 PUFA ratio in $CD4^+$ T cells was found to be regulated primarily by the dietary n-3 PUFA content. Based on the present results, we consider reduced signal transduction through the TCR and CD28 as a key contributor to the reduced proliferation and costimulatory molecule expression found in n-3 PUFA-rich $CD4^+$ T cells.

We demonstrated here that the strength of the DC signal plays a distinct regulatory role in the proliferation and expression of costimulatory molecules in $CD4^+$ T cells. The strength of the DC signal is regulated by all microbes, and thus also by viruses, parasites, fungi and pathogenic bacteria, and is based on their carriage of microbe-associated molecular patterns. The present data are therefore not only valid for gut bacteria-induced priming of DCs, but may describe a general down-regulatory mechanism of dietary and cellular n-3 PUFAs on $CD4^+$ T-cell responses. In this study, we used different generas and concentrations of gut bacteria to prime the DCs to display different levels of the surface markers MHC class II, CD80, CD86 and CD40, as also previously reported by our group.[27,31] This approach enabled us to perform correlations between DC surface display and $CD4^+$ T-cell activation. Significant correlations were found between the expression level of CD80 on DCs and the inverse percentage of CD28-positive cells on the proliferation ability of $CD4^+$ T cells, emphasizing that the intensity of the CD80 signal from DCs has a greater effect on cell proliferation (via CD28 costimulation) than the magnitude of MHC class II, CD86 or CD40 signalling. Conversely, CD40 and CD86 expression levels on DCs were found to correlate inversely with the percentage of ICOS-positive $CD4^+$ T cells and also with the percentage of CTLA-4-positive $CD4^+$ T cells. Similar regulatory control of CD40, ICOS-L (B7RP-1) and CD86 on primed DCs may explain this finding, as there is no evidence for CD40 and ICOS or CTLA-4 to directly interact. Indeed, the correlations between CD28 and CD80 expression levels, as well as between the CTLA-4 and CD86 expression levels, add to the current knowledge on the effect of B7 molecules on DCs for regulation of CD28 and CTLA-4. In the present study we did not measure ICOS-L, but our data on CD40 and CD86 expression on DCs shows a similar pattern of CD86 and CD40 regulation in DCs (Fig. 3b). Yet others have reported high expression levels of ICOS-L on immature DCs, and down-regulation upon stimulation with LPS, thus implying that ICOS-L is regulated inversely to CD40 and CD86.[32] In regard to $CD4^+$ T-cell costimulatory molecules, it was previously demonstrated that co-regulation of costimulatory molecules is occurring (e.g. ICOS costimulation involving up-regulation of CD40L on T cells).[17] Based on our findings here, it would be of interest to pay specific attention to the mechanistic regulation of these molecules on DC and $CD4^+$ T cells, but as the influence of the cellular lipid content was our main focus in this study, this aspect was not further studied. The inverse association found to be present between CD40 and CD86 on DCs, and between ICOS and CTLA-4 on $CD4^+$ T cells could, to some extent, be caused by an activation-induced internalization of $CD4^+$ ligands upon DC ligand interaction, as previously reported for CD28,[29] and for B-cell-expressed ICOS-L after interaction with ICOS transfectant cell lines,[21] but cannot be the sole mechanism, as revealed

from the CTLA-4 measurements. CTLA-4 was measured simultaneously both intracellularly and at the surface, and therefore the total lower percentage of CTLA-4-positive cells after stimulation with DCs expressing high levels of CD40 and CD86 cannot result from internalization of CTLA-4 only; rather *de novo* synthesis of the molecule may be reduced. As CTLA-4 is recognized to be induced in cells in order to down-regulate $CD4^+$ T-cell activation,[24] the lower percentages of CTLA-4 in $CD4^+$ T cells after potent stimulation by DCs may be a direct result of enhanced activation of the cell, and thus delayment of CTLA-4 expression. In relation to the down-regulation of ICOS-positive cells with strong DC stimulation, we are, however, not able to come up with any firm conclusions, as most current literature indicates that ICOS is important for positive costimulation. Yet, the most likely reason for the ICOS expression pattern is activation-induced internalization of the ligand, as also assumed to be the case for CD28. This is strongly supported by the proliferation data showing that the most potent DC stimuli induce the highest proliferative probability in $CD4^+$ T cells, thus clearly signifying that the $CD4^+$ T cells with the lowest percentage of surface CD28 and ICOS expression are the ones being activated most.

The data reveal a dichotomy between the down-regulation of the percentage of CD28-positive and ICOS-positive cells by potent DC stimuli, presumed to indicate higher $CD4^+$ T-cell activation, and the lower number of ICOS-positive cells and CTLA-4-positive cells in n-3 PUFA-rich cells, which, by using the same assumption, also might be ascribed to even higher activation. However, as proliferation ability is lowered in n-3 PUFA-rich cells, and because the percentages of ICOS-positive and CTLA-4-positive cells after stimulation with iDCs are lower in n-3 PUFA-derived cells than in SFA-derived cells, we imply that this duality is caused by a general reduction of TCR and CD28 signalling in n-3 PUFA-rich cells. Diminished TCR and CD28 signalling will give rise to lower levels of CTLA-4 and ICOS expression in DC-activated $CD4^+$ T cells, as both costimulators are recognized to be regulated by TCR activation and further enhanced by CD28 stimulation.[29,33] In turn, this also results in reduced proliferation ability in the n-3 PUFA-rich group. Still, independently of the differences in signal transmission seen between the two dietary groups, the strength of the DC signal was equally important for the resulting activation level of the $CD4^+$ T cells in terms of proliferation and costimulatory molecule expression, emphasizing that the n-3 PUFA-rich $CD4^+$ T cells are indeed sensing the differences in DC signal strength, despite being less responsive.

One probable explanation for the observed reduction in $CD4^+$ T-cell reactivity might be disruption of raft integrity, as n-3 PUFAs are reported to incorporate into raft lipids,[2] leading to displacement of lipid raft-associated proteins involved in both TCR- and CD28-mediated signalling (e.g. LAT and PKCθ), [2,34] but several other mechanisms may also play a role. CD28 is constitutively expressed at constant levels on naïve cells, and is recruited to lipid raft regions during T-cell activation.[35,36] In the event of lipid raft disruption by some n-3 PUFAs, it will therefore not be surprising that we observed a general down-regulation of DC-induced, as well as anti-CD3 and anti-CD28-induced, $CD4^+$ T-cell activation in cells high in n-3 PUFAs. Lipid raft-mediated effects of n-3 PUFAs would also explain the diversity in production of IL-5 versus IFN-γ and IL-10 seen in activated $CD4^+$ T cells, as IL-5 transcription was previously reported not to involve LAT activation.[37] No effect of the cellular FA composition was found in regard to CD28 surface expression in resting $CD4^+$ T cells, thus excluding a direct regulatory effect of cellular PUFAs on CD28 surface display. This latter finding is in agreement with a previous report from Ly *et al.*[14]

Both ICOS and CTLA-4 are important for regulation of acquired immunity, and a lower percentage of activation-induced ICOS- and CTLA-4-positive cells in the n-3 PUFA group is therefore proposed to directly influence the propensity of $CD4^+$ T cells to activate and regulate adaptive immune responses. Enhanced ICOS expression has recently been linked to some autoimmune diseases, such as rheumatoid arthritis,[20] a disease in which patients usually benefit from n-3 PUFA supplementation.[38] It is plausible that the effect of n-3 PUFA in patients with rheumatoid arthritis is caused by the reduction in ICOS expression on $CD4^+$ T cells induced by the presence of cellular n-3 PUFA; however, multiple mechanisms are probably involved, including the general reduction of $CD4^+$ T-cell responsiveness in cells high in n-3 PUFAs. In contrast to ICOS and CD28, CTLA-4 serves as a negative regulator of T-cell responses,[1] being indispensable in preventing fatal lymphoproliferative disease,[39] which emphasizes that CTLA-4 is essential for maintaining immune homeostasis. Upon T-cell activation, CTLA-4 is expressed on the surface within 2–3 days after activation, but, because of rapid endocytosis from the cell surface, the majority of protein is present in intracellular vesicles.[30] Our data on diminished CTLA-4-levels in n-3 PUFA-rich stimulated cells indicates that CTLA-4 is not actively involved in the reduced proliferation observed for $CD4^+$ T cells high in n-3 PUFAs, as formerly suggested by Ly *et al.*[14]

Despite several previous studies identifying specific cellular mechanisms affected by, especially, n-3 PUFAs in regard to T-cell responsiveness, the consequence of the dietary n-6 : n-3 PUFA ratio for general T-cell activation is still incompletely defined. In cell types other than immune cells, the cellular n-3 PUFA and n-6 PUFA membrane levels were previously reported to highly reflect the dietary composition.[40] Conversely, membranes

remain relatively constant in their SFA and MUFA content over a wide range of dietary variations for these fatty acids.[40] We found that this was indeed also the case for $CD4^+$ T cells, emphasizing that the dietary n-6 : n-3 PUFA ratio, rather than the SFA, MUFA or absolute n-6 PUFA intake, influences the cellular lipid composition.

Collectively, our data showed that the dietary n-6 and n-3 PUFA contents regulate the cellular n-6 : n-3 PUFA ratio, with a high n-6 : n-3 PUFA ratio being essential for $CD4^+$ T cells to respond strongly to external signals (e.g. provided by bacteria-primed DC). Incorporation of cellular n-3 PUFAs is central in regard to reduced signal transduction through the TCR and CD28. A deregulation of signalling through the TCR and CD28 was found to affect the modulation of other T-cell costimulatory molecules, such as ICOS and CTLA-4, demonstrated here to be reduced upon the presence of cellular n-3 PUFA at the same time as being regulated by the DC signal strength. Importantly, the presence of cellular n-3 PUFA does not prevent $CD4^+$ T-cell activation but rather leads to reduced responsiveness, which, in terms of aberrant immune reactions such as autoimmunities and allergies, might decrease the propensity of antigen-specific $CD4^+$ T cells to become activated by requiring a generally higher threshold of stimulation in order to induce immune activation. In addition to the present and previously revealed effects of n-3 PUFA on $CD4^+$ T cells, dietary PUFAs have previously been shown to affect the function of DCs and other APCs, giving rise to reduced expression of surface molecules and diminished cytokine production.[12] Thus, during *in vivo* conditions, DC-induced activation of $CD4^+$ T cells high in n-3 PUFAs might be reduced even more than currently revealed here, where we used *in vitro* stimulation with DCs that had identical lipid compositions.

Conclusively, the present findings demonstrate that the activation of $CD4^+$ T cells clearly depends upon the strength and the type of the DC signal, but that the responsiveness of cells is reduced in n-3 PUFA-rich cells, thus indicating that changes in the dietary n-3 PUFA content play a central role in the overall ability of $CD4^+$ T cells to respond to an exogenous signal provided by DCs.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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